Enzyme-mediated synthesis of two diastereoisomeric forms of phosphatidylglycerol and of diphosphatidylglycerol (cardiolipin)

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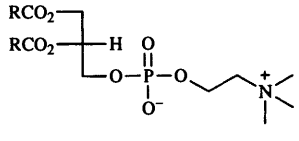
3-*sn*-Phosphatidyl-1'-*sn*-glycerol and its stereoisomer 3-*sn*-phosphatidyl-3'-*sn*-glycerol are prepared through phospholipase D-catalysed transphosphatidylation of natural phosphatidylcholine with the two enantiomeric (*R*)- and (*S*)-isopropylideneglycerols and subsequent hydrolysis of the phospholipids obtained. If glycerol is the alcohol donor in the same reaction, a mixture of stereoisomers is obtained. When the enzyme from savoy cabbage is used, the two compounds are obtained as the only products. With PLD from *Streptomyces* as catalyst, the initially formed phosphatidylglycerols are quantitatively transformed into diphosphatidylglycerol (cardiolipin).

Introduction

Glycerophospholipids (PL) are widely distributed in Nature as mixtures of compounds differing in their polar head and the peculiar fatty acid composition according to their origin.[1] Purification from natural mixtures is usually convenient for the most abundant component, *i.e.* phosphatidylcholine (PC) (1,2-diacyl-*sn*-glycero-3-phosphorylcholine) **1**, while other glycerophospholipids with a defined polar head and/or acyl chains are better prepared by synthesis or semisynthesis.[2]



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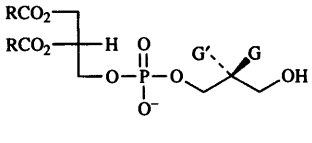
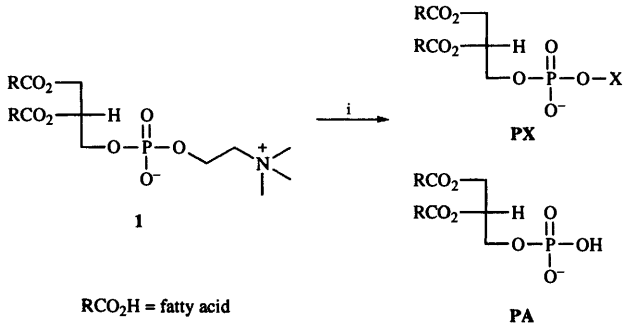


2a G = OH, G' = H
b G = H, G' = OH

The availability of enzymes with a wide substrate specificity and high transfer capacity offers a very convenient access to modified natural PLs. The application of phospholipase D (PLD)[3] from bacterial sources allows the highly selective transfer of a nucleophilic component (alcohol) in a water medium to the PL (usually PC) with the formation of a new phospholipid PX[4] and of the acid PA in different ratios depending upon the nature of the enzyme, the type and concentration of the alcohol and the experimental conditions (Scheme 1). We

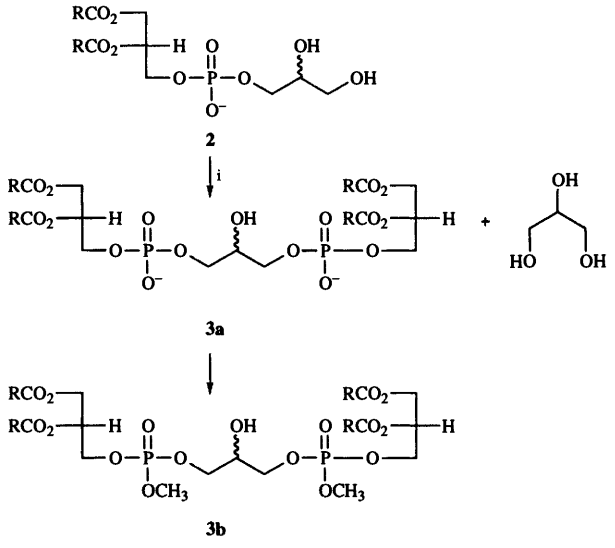


1 **PX** **PA**

RCO_2H = fatty acid

Scheme 1 *Reagents:* XOH, water, PLD

now report on the application of PLD to the practical preparation of the two diastereoisomeric phosphatidylglycerols (PG), namely 3-*sn*-phosphatidyl-1'-*sn*-glycerol **2a** and 3-*sn*-phosphatidyl-3'-*sn*-glycerol **2b** and of 1',3'-bis(1,2-diacyl-*sn*-glycero-3-phosphoryl)glycerol (diphosphatidylglycerol, cardiolipin, CL) **3a** (Scheme 2).



2 **3a** **3b**

Scheme 2 *Reagent:* i, PLD

Results and discussion

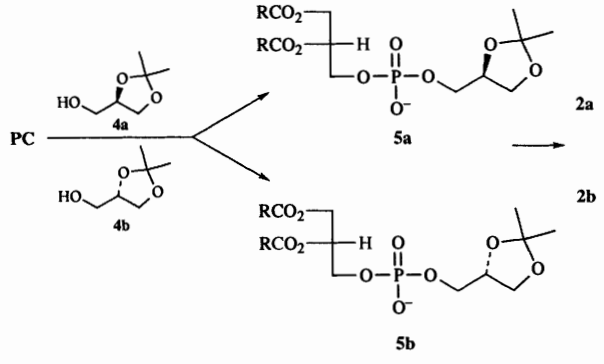
Preparation of PGs. Stereochemistry of PLD-catalysed transphosphatidylation

Phosphatidylglycerol **2a** is one of the four or five more abundant phospholipids found in natural membranes. It possesses the absolute configuration shown in structure **2a**, as shown by degradation with phospholipase C and submitting the glycerol phosphate thus obtained to *sn*-glycerol-3-phosphate dehydrogenase.[5] The preparation of this compound from PC by transphosphatidylation catalysed by PLD from savoy cabbage has been reported.[4h] When glycerol is used in a 10 M concentration, the concomitant formation of phosphatidic acid is suppressed, allowing the obtainment of PG as the sole product. The configuration of the newly formed chiral centre has been

the subject of much debate.[6] It has finally been shown by experiments with labelled material that indeed the product formed is a 1 : 1 mixture of the two diastereoisomers.[6d] Commercially available PG, which is produced in this way, is a mixture of the two stereoisomers. The lack of enantioselectivity of PLD from savoy cabbage seems common to other PLDs from bacterial sources. Modest recognition has been observed in the reaction of PC with racemic prolinol with a commercial PLD from *Streptomyces*,[4f] while D-serine has been reported to be transferred with preference to L-serine.[7] When we used PLD from *Streptomyces sp.*[8] and glycerol, the obtained PG was a mixture of stereoisomers in a ratio near to 1 : 1. This was confirmed by the procedure described below by which we were able to secure an easy access to both PGs with the natural configuration in the *sn*-2 position. This was possible because of the relaxed substrate specificity shown by PLD from the above source.†

Synthesis of the two stereoisomeric PGs with the natural configuration at the *sn2* position

Reaction of PC with (*R*)- and (*S*)-solketals, **4a** and **4b**, respectively, in a biphasic system in the presence of PLD from *Streptomyces sp.* afforded the corresponding phosphatidylsolketals **5a** and **5b** in 78–85% HPLC yield before purification (Scheme 3). The two alcohols are excellent substrates for the



Scheme 3

enzyme. When they are used in a 1 M solution in the presence of PC with 80 U of enzyme per g of phospholipid, the reaction is complete in 35 min with less than 2% formation of PA. The two stereoisomeric compounds could be easily differentiated from their ^{1}H NMR spectra. Hydrolysis in aq. MeOH in the presence of toluene-*p*-sulfonic acid (PTSA) afforded the two PGs **2a** and **2b** in quantitative yield (Scheme 3). This procedure can be applied on a 50 g scale in the laboratory. The PG obtained from PC and glycerol *via* transphosphatidylation with PLD was ketalised with 2,2-dimethoxypropane–acetone and acid catalysis to give a phospholipid, which by ^{1}H NMR studies proved to be a near 1 : 1 mixture of the stereoisomers prepared *via* the solketal procedure. Previous synthesis of the two PG required tedious sequences of protection/deprotection steps leading invariably to very low yields.[9]

Synthesis of cardiolipin

During the preparation of PG from PC and glycerol, we realised that the enzyme from savoy cabbage gave a product of higher purity compared to the one obtained when we used the bacterial enzyme. Indeed if the initially formed PG is stirred in the presence of the enzyme, it is slowly converted into diphosphatidylglycerol (cardiolipin). In this case one molecule of PG acts as the alcohol donor for a second one, thus giving rise to the observed CL (Scheme 2). After reaction for 5 days at 28 °C, all the PG was transformed into CL **3a**.‡ PA was formed only in small amounts (10%). This reaction can be easily performed on a preparative scale. Purification of the calcium salt by silica gel chromatography gave a product of good purity, identical with a commercially available specimen and in spectral data with the literature.[10,11] Conversion into the dimethyl ester **3b** through a known procedure confirmed the structure of the compound.[11]

Cardiolipin is a lipid component of all cell membranes. It is one of the three major phosphoglycerides in the *Escherichia coli* cell envelope and is particularly abundant in mammalian heart.[12] It has been shown to be the essential lipid of the antigen in serodiagnosis of syphilis.[13] Its formation *in vivo* follows at least two different pathways: it derives from cytidine-5′-diphosphate (CDP) diglyceride and *sn*-glycerol-3-phosphate in liver mitochondria[14] and from PG alone in *E. coli* not requiring citidine activation, as shown by using a cell-free extract from *E. coli* and precursors with radioactive labels.[15] Similar experiments were also employed to show the formation of CL from PG with cabbage PLD.[16] In the latter case, however, CL was present only in trace amounts. The present synthesis is therefore the first preparative approach to CL based on a total enzymic transformation of PC. This methodology is not confined to PC of natural origin but has been applied on PC with defined acyl chains with similar success.[17] Chemical synthesis of CL can have the advantage over the enzymic approach of allowing the preparation of compounds bearing different acyl chains in the two glycerophosphoryl halves.[11] The total-synthesis approach is, however, rather complex for large-scale preparations. In comparing the kinetic data for the formation of CL from the two PGs only a small difference in the initial velocity was observed. The high overall selectivity is due to the fact that both competing reactions, *i.e.* CL hydrolysis to PG and PA and the formation of 2PG from 1CL + glycerol, are extremely slow or not measurable at all. Cardiolipin is hydrolysed in *Haemophilus parainfluenzae* by a cardiolipin-specific PLD. The mechanism of action of this enzyme, in particular the site of hydrolysis, has been investigated.[18] A similar capacity is apparently not present in the PLD used in our investigation.

Conclusions

PLD from *Streptomyces sp.* efficiently catalyses the transfer of (*R*)- and (*S*)-solketal with PC, giving the two phosphatidylsolketals in good yields and purity. Their hydrolysis gives access to the two stereoisomeric PGs with the natural configuration at position *sn*-2. This material was previously not accessible on a gram scale. Comparison of the PGs obtained by direct reaction of PC with glycerol using different PLD preparations confirms that the reaction occurs without enantioselectivity. PLD from bacterial sources is further able to transform PG of either diastereoisomeric composition to diphosphatidylglycerol (cardiolipin) in good yields.

Experimental

Natural PC was from Lucas-Meyer AG (FRG). (*R*)- and (*S*)-Solketal were from Italfarmaco Sud (Italy). Cabbage PLD was from Boehringer-Mannheim and PLD(P) was from Asahi (Japan). HPLC analyses were performed on a Merck Hitachi L-6200 with a Supelcosil LC-Si 5μ column, 25 cm × 4.6 mm (Supelco) and UV (206 nm) detector using a Merck Hitachi D-2500 integrator. HPLC analysis conditions were: eluent hexane–Pr^{i}OH–NaOAc buffer 0.2 M, pH 4.2 = 8 : 8 : 1; flow 2 ml min^{-1}. The microorganism was grown as previously reported.[8] The PLD activity was measured with a colorimetric assay.[19] 1 U

† Some of the PLDs from other bacterial sources behave in a similar way. PLD(P) from Asahi (Japan) is similar in this respect.

‡ During CL preparation every second day the water phase was separated and replaced with a new solution with fresh enzyme.

of activity is defined as the amount of enzyme hydrolysing one μmol of phosphatidyl-*p*-nitrophenol per min at 37 °C.

Samples of PG and CL were obtained from Sigma. Analytical samples of compounds **2**, **3** and **5** were prepared by flash chromatography on silica gel with mixtures of CH_2Cl_2–MeOH. All compounds were isolated as their Ca^{2+} salts unless otherwise stated.

Silica gel 60 F254 plates (Merck) were used for analytical TLC. Developers used: $CHCl_3$–CH_3OH–NH_3 = 65:30:2.5; $CHCl_3$–CH_3OH–CH_3COCH_3–AcOH–water = 50:10:20:10:5. Phospholipids were detected by spraying with phosphomolybdic acid, followed by heating.

^1H, ^{13}C NMR spectra were recorded on a Bruker AC-250 or a Bruker CPX-300 instrument with tetramethylsilane as internal standard. All spectra were recorded in $CDCl_3$–CD_3OD = 2:1. *J*-Values are given in Hz. FAB spectra were run with a Finnigan Mat 70-70 TSQ triple-quadrupole instrument equipped with xenon as bombarding beam operating in negative ion mode using thioglycerol or tetraethylene glycol matrices. Ions corresponding to the 18:2–16:0 $M_1 = [M - H]^-$ and 18:2–18:2 $M_2 = [M_1 + 24]^-$ combination of acyl chains are reported. Optical rotations were measured on a Propol digital polarimeter; $[a]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}.

3-*sn*-Phosphatidylglycerol 2

PC (10 g) was dissolved in 100 ml of CH_2Cl_2 and mixed with 200 ml of water containing 1.64 g of NaOAc (0.1 M), 146 g of glycerol (8 M), 2.22 g of $CaCl_2$ (0.1 M) and 300 U of PLD from *Streptomyces sp.*,[8] and the final pH was adjusted to 5.6 with acetic acid. The mixture was stirred with a mechanical stirrer at 200 rpm at 25 °C. Occasionally an aliquot was withdrawn and analysed by HPLC. After 7 h the reaction was complete. The organic phase was washed several times with water (50 ml). The dried organic phase gave a residue (13 g). The mixture was purified by flash chromatography with mixtures of CH_2Cl_2–MeOH to give 9.5 g of compound **2** as a waxy solid. TLC of the crude product showed a spot corresponding to cardiolipin, which was not present after purification; δ_H 0.89 (t, terminal CH_3), 1.3 (m, aliphatic protons), 1.6 (m, 4 H, CH_2CH$_2$CO$_2$), 2.05 (8 H, allylic CH_2), 2.35 (4 H, CH_2CO_2), 2.75 (4 H), 3.7–4.5 (m) and 5.3–5.45 (m, olefinic protons); δ_C 14.18 (terminal CH_3), 22.92–34.46 (aliphatic CH_2), 62.57 (glycerol 3′-CH) 62.98 (glycerol 1-CH_2), 63.97 (glycerol 3-CH_2), 66.77 (glycerol 1′-CH), 70.64 (glycerol 2′-CH), 71.24 (glycerol 2-CH), 128.14, 128.36, 130.17 and 130.43 (olefinic C=C) and 173.92 and 174.18 (C=C); MS M_1, 729.5 and M_2, 753.5.

3-*sn*-Phosphatidylsolketal 5a

PC (6 g) and 17 g of (*R*)-solketal **4a** were dissolved in 45 ml of CH_2Cl_2 and mixed with 130 ml of water containing 1.05 g of NaOAc (0.1 M), 1.4 g of $CaCl_2$ (0.1 M) and 180 U of PLD from *Streptomyces sp.*,[8] and the final pH was adjusted to 5.6 with acetic acid. The mixture was stirred with a mechanical stirrer at 200 rpm at 37 °C and the reaction was followed by HPLC. After 35 min the reaction was complete. The organic phase was washed with water (50 ml). The crude material was shown to contain 87% of the new phospholipid and 10% of PA. The mixture was purified by flash chromatography with mixtures of CH_2Cl_2–MeOH to give 4.5 g of compound **5a** as a waxy solid, $[a]_D^{20}$ +4.5 (*c* 1, $CHCl_3$); δ_H 0.88 (terminal CH_3), 1.25–1.45 (m, aliphatic CH_2), 1.7 (CH_2CH$_2$CO), 2.1 (allylic CH_2), 2.35 (m, CH_2CO), 2.8 (4 H, m), 3.77–3.92 (3 H, m), 3.98 (2 H, t, *J* 6), 4.06 (1 H, dd, *J* 6.25, 8.5), 4.18 (1 H, dd, *J* 7, 12), 4.28 (1 H, quintet, *J* 6), 4.41 (2 H, dd, *J* 3, 12), 5.25 (1 H, m, CHOCO) and 5.3–5.4 (m, olefinic protons); δ_C 14.33 (terminal CH_3), 21.05–25.45 (aliphatic CH_2), 25.53 (CH_3 of solketal), 26.02–26.14 (aliphatic CH_2), 26.97 (CH_3 of solketal), 27.73–34.73 (aliphatic CH_2), 63.30 (glycerol 1-CH_2), 64.23 (glycerol 3-CH_2), 66.6 and 66.90 (polar head CH_2), 71.22 (glycerol 2-CH), 75.3 (polar head CH), 110.10 (solketal quaternary C), 128.45, 128.63, 130.18 and 130.36 (olefinic C=C) and 174.05 and 174.41 (C=O); MS M_1, 785.5 and M_2, 809.5.

3-*sn*-Phosphatidylsolketal 5b

PC (6 g) and 17 g of (*S*)-solketal **4b** were dissolved in 45 ml of CH_2Cl_2 and mixed with 130 ml of water containing 1.05 g of NaOAc (0.1 M), 1.44 g of $CaCl_2$ (0.1 M) and 180 U of PLD from *Streptomyces sp.*, and the final pH was adjusted to 5.6 with acetic acid. The mixture was stirred with a mechanical stirrer at 200 rpm at 37 °C and the reaction was followed by HPLC. After 35 min the reaction was almost complete. The organic phase was washed with water (50 ml). The crude material was shown to contain 93% of the new phospholipid, 2% of PA, and 2.5% of PC. The mixture was purified by flash chromatography with mixtures of CH_2Cl_2–MeOH to give 4.8 g of compound **5b** as a waxy solid, $[a]_D^{20}$ +5.2 (*c* 1, $CHCl_3$); δ_H 0.88 (terminal CH_3), 1.25–1.4 (m, aliphatic CH_2), 1.6 (CH_2CH$_2$CO), 2.05 (allylic CH_2), 2.35 (m, CH_2CO), 2.8 (4 H, m), 3.80–3.92 (3 H, m), 3.98 (2 H, t, *J* 6), 4.065 (1 H, dd, *J* 6.25, 8.5), 4.175 (1 H, dd, *J* 7 and 12), 4.275 (1 H, quintet, *J* 6), 4.43 (2 H, dd, *J* 3, 12), 5.25 (1 H, m, CHOCO) and 5.3–5.4 (m, olefinic protons); δ_C 14.21 (terminal CH_3), 22.85–25.19 (aliphatic CH_2), 25.38 (CH_3 of solketal), 25.79–25.90 (aliphatic CH_2), 26.82 (CH_3 of solketal), 27.48–34.49 (aliphatic CH_2), 63.02 (glycerol 1-CH_2), 64.01 (glycerol 3-CH_2), 66.40 and 66.66 (polar head CH_2), 70.87 (glycerol 2-CH), 74.99 (polar head CH), 109.86 (solketal quaternary C), 128.39, 128.47, 130.19 and 130.43 (olefinic C=C) and 173.79 and 174.13 (C=O); MS M_1, 785.5 and M_2, 809.5.

3-*sn*-Phosphatidyl-1′-*sn*-glycerol 2a

3-*sn*-Phosphatidylsolketal **5a** (4.2 g) was dissolved in 7 ml of CH_2Cl_2 and diluted with 150 ml of MeOH to give a cloudy solution. The mixture was treated with 25 ml of water and 200 mg of PTSA and was refluxed for 30 min. The mixture was then evaporated to dryness, the residue was dissolved in 20 ml of CH_2Cl_2, and the solution was washed successively with brine (20 ml) and saturated aq. $NaHCO_3$. The organic phase was dried and evaporated to give compound **2a** (3 g), $[a]_D^{20}$ +3 (*c* 5, $CHCl_3$).

With exactly the same procedure, starting with compound **5b**, compound **2b**, $[a]_D^{20}$ +6.6 (*c* 5, $CHCl_3$), was obtained.

3-*sn*-Phosphatidylsolketal (5a + 5b) from 3-*sn*-phosphatidylglycerol 2

Compound **2** (2.5 g) was dissolved in a mixture of 2,2-dimethoxypropane (20 ml) and acetone (5 ml) and the solution was treated with PTSA (200 mg). The mixture was stored at room temperature for 10 h. The solvent was then removed under reduced pressure and the crude material was purified by flash chromatography to give 1.5 g of phosphatidylsolketal, which from ^1H NMR studies proved to be a mixture of compounds **5a** and **5b**. The ratio of the two compounds was evaluated by an examination of the signals at δ_H 4.15–4.3 and by comparison with artificial mixtures of the two compounds **5a** and **5b**. The product was estimated to be a 1:1 mixture with a possible error of 10%.

1′,3′-Bis(1,2-diacyl-*sn*-glycero-3-phosphoryl)glycerol 3a (cardiolipin)

PG (3 g) was dissolved in 60 ml of CH_2Cl_2 and mixed with 120 ml of water containing 0.98 g of NaOAc (0.1 M), 1.33 g of $CaCl_2$ (0.1 M) and 180 U of PLD from *Streptomyces sp.* and the final pH was adjusted to 5.6 with acetic acid. The mixture was stirred with a mechanical stirrer at 200 rpm at 37 °C. Occasionally an aliquot was withdrawn, and analysed by HPLC. After 24 h the mixture was allowed to separate into the two phases. The water phase was withdrawn and replaced with an identical one with fresh enzyme. After 5 days the reaction was complete. The organic phase was separated, dried and the residue of 3.5 g was purified by flash chromatography with mixtures of CH_2Cl_2–

MeOH to give 2.2 g of cardiolipin **3a** as a waxy solid; δ_H 0.88 (terminal CH_3), 1.3 (m, aliphatic CH_2), 1.62 (CH_2CH_2CO), 2.18 (allylic CH_2), 2.32 (8 H, m, CH_2CO), 2.8 (4 H, m), 4.0–4.5 (m), 5.25 (2 H, m, CHOCO) and 5.3–5.5 (m, olefinic protons); δ_C 14.24 (terminal CH_3), 22.88–34.50 (aliphatic CH_2), 63.02 (glycerol 1-CH_2), 64 (glycerol 3-CH_2), 66 (CH_2OPO_2), 70.86 (glycerol 2-CH), 70.99 (CH on the polar head), 128.20, 128.40, 130.22 and 130.48 (olefinic C=C) and 173.86 and 174.21 (C=O).

Cardiolipin dimethyl ester 3b

Crude cardiolipin **3a** (1.2 g) was dissolved in 150 ml of $CHCl_3$–MeOH–water = 2:1:0.2. The solution was washed with 20 ml of cold 0.1 M HCl and $CHCl_3$–MeOH–water (0.1:1:1). The organic layer was concentrated to a small volume and treated with an excess of ethereal diazomethane for 30 min at 0 °C. The residue was purified by preparative TLC and identified as the dimethyl ester **3b**; δ_H 0.88 (terminal CH_3), 1.25 (m, aliphatic CH_2), 1.6 (CH_2CH_2CO), 2.2 (allylic CH_2), 2.3 (8 H, m, CH_2CO), 2.8 (4 H, m), 3.75 (d, $POCH_3$), 3.8 (d, $POCH_3$), 4.1–4.4 (m), 5.25 (2 H, m, CHOCO) and 5.3–5.5 (m, olefinic protons).

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